Improved Fc Fusion Proteins

Description

The invention relates to fusion proteins comprising at least a biologically active polypeptide domain and a second domain selected from a constant immunoglobulin domain.

Fusion proteins comprising an immunoglobulin heavy and/or light chain dimer or an immunoglobulin heavy and/or light chain tetramer, in which an amino acid sequence of a ligand-binding partner which is a receptor, a carrier protein, a hormone, a growth factor or an enzyme, is substituted for the variable region of at least one immunoglobulin chain, are described in EP-A-0 526 452. A fusion protein comprising the extra cellular domain of the death receptor CD95 (APO-1; Fas) fused to an immunoglobulin Fc fragment is described in WO 95/27735. N-terminally truncated derivatives of the APO-1 molecule optionally fused to immunoglobulin Fc fragments are disclosed in EP-A-0 965 637. A fusion protein consisting of soluble IL-15Rα and Fc fragments is disclosed in WO 98/36768. A fusion protein consisting of an antagonist IL-15 mutant and an Fc IgG2a fragment is disclosed by Kim et al. (*J. Immunol.* 160 (1998), 5742-5748). These documents are incorporated herein by reference.

Although it has been shown that fusion proteins as described above have high biological activity *in vitro* and *in vivo,* there are concerns with regard to the immunogenic potential of such molecules since there is a fusion region between two protein domains of different origin comprising a non-naturally ocurring amino sequence which may elicit an undesired immune response in an organism to which the fusion protein is administered.

WO 02/066514 describes artificial fusion proteins having a reduced immunogenicity compared to the parent non-modified molecule when exposed to a species *in vivo*. These proteins essentially consist of an

immunoglobulin molecule or a fragment thereof covalently fused via its C-terminus to the N-terminus of a biologically active non-immunoglobulin molecule, preferably a polypeptide or protein or a biologically active fragment thereof. The molecules have amino acid sequences which are altered in one or more amino acid residue positions but, in principle, have the same biological activity as compared with the non-altered molecules. The changes are made in regions of the molecules which are identified as T-cell epitopes, which contribute to an immune reaction in a living host. A disadvantage of this procedure, however, is that not all epitopes, particularly not B-cell epitopes, can be reliably eliminated. Furthermore, the introduction of non-naturally occurring amino acid sequences can lead to the generation of neo-epitopes.

Thus, it was an object of the present invention to provide fusion proteins with at least two domains of different origin having a reduced immunogenic potential.

Thus, the present invention relates to a fusion protein comprising
(i) at least one first domain comprising a biologically active polypeptide and
(ii) a heterologous second domain comprising at least a portion of a constant immunoglobulin domain,
wherein there is at least one amino acid overlap between the first domain and the second domain in the fusion region.

The fusion protein may be a monomeric protein or a multimeric protein, e.g. a dimeric or tetrameric protein, which may be formed by multimerisation via the constant immunoglobulin domain.

According to the present invention, the design of a fusion protein comprises i) the selection of at least one first domain and a second domain which is heterologous to the first domain and ii) the selection of at least one terminal amino acid which is common to the first and the second domain, e.g. the last amino acid(s) of the first domain is (are) selected such that they are identical

with the first amino acid(s) of the second domain. Preferably, the overlap has a length of one, two or three amino acids. Thus, a fusion protein is obtained which is free from a non-naturally occurring transition between the last amino acid of one domain and the first amino acid of another domain.

In an embodiment of the invention, the first domain(s) is (are) located at the N-terminus of the fusion protein, whereas the second domain is located at the C-terminus. Thus, in this embodiment, at least one carboxy terminal amino acid of a first domain overlaps with at least one amino terminal acid of the second domain.

In a further embodiment the second domain is located at the N-terminus of the fusion protein and the first domain(s) is (are) located at the C-terminus. Thus, in this embodiment, at least one carboxy terminal amino acid of the second domain overlaps with at least one amino terminal acid of a first domain.

In cases where the fusion protein comprises more than one, e.g. two or three, first domains, these domains are preferably located sequentially at the N-terminus or the C-terminus of the fusion protein and the second domain at the C-terminus or at the N-terminus, respectively. It should be noted that the first domains in such proteins may be the same or different. Transitions between individual first domains are preferably designed such that there is also at least one amino acid overlap (and thus not a non-naturally occurring transition between the last amino acid of one domain and the first amino acid of the other domain) between the individual first domains. Fusion proteins comprising multiple first domains are disclosed in WO 00/18932 which is incorporated herein by reference.

The first domain of the fusion protein comprises a biologically active polypeptide, i.e. a polypeptide which is capable of interacting with, e.g. binding to, a binding partner, e.g. another polypeptide, in its natural environment in a cell or an organism and which is preferably capable of

exhibiting a pharmacological activity. The first domain is preferably a non-immunoglobulin polypeptide. The first domain may be a naturally occurring polypeptide or a variant thereof having desired, e.g. increased or reduced, biological activity or a fragment of a naturally occurring polypeptide or a variant thereof. The first domain is preferably selected from the ligand-binding domain of a receptor and a receptor-binding domain of a ligand. The terms „ligand" and „receptor" are understood in this context such that ligands are defined as proteins known to function to bind specifically to receptor molecules. The term „receptor" includes soluble or membrane-anchored receptor proteins having a hydrophobic transmembrane region or a phospholipid anchor. Further, the term „receptor" encompasses carrier proteins as well as hormones, cellular adhesive proteins, lectins, growth factors, enzymes, etc.

In a preferred embodiment of the invention the first domain is a ligand-binding receptor domain comprising the extra-cellular domain of a membrane-anchored receptor or a ligand-binding fragment thereof. The receptor is preferably selected from death receptors, growth factor receptors and cytokine receptors. More preferably, the receptor is selected from CD95 (APO-1; Fas), TRAIL receptors, TNF receptors, VEGF receptors, an interleukin receptor such as IL-15Rα. Most preferably the receptor is CD95, a TRAIL receptor, e.g. the TRAIL receptor-1, the TRAIL receptor-2, the TRAIL receptor-3 or the TRAIL receptor-4 or a TNF receptor, e.g. the TNF receptor-1 or the TNF receptor-2.

In a further embodiment, the first domain is a receptor-binding ligand domain. The ligand is preferably selected from death ligands such as the CD95 ligand, TRAIL, TNF, e.g. TNF-α or TNF-β, growth factors, e.g. VEGF and cytokines, such as interferons or interleukins, e.g. IL-15 or variants thereof.

In a still further embodiment, the fusion protein comprises multiple first domains which may be the same or different. A preferred example of such a

multiple fusion protein is a VEGF Trap fusion protein comprising the second extracellular domain of the VEGF receptor 1 (Flt-1) with the third domain of the VEGF receptor 2 (KDR/FIK-1) and an IgG constant region.

The first domain protein is preferably a mammalian protein, more preferably a human protein. For therapeutic purposes in particular, the use of human proteins is preferred.

The second domain of the fusion protein comprises at least a portion of a constant immunoglobulin domain, e.g. a constant heavy immunoglobulin domain or a constant light immunoglobulin domain. Preferably, the second domain comprises at least a portion of a constant heavy immunoglobulin domain. The constant heavy immunoglobulin domain is preferably an Fc fragment comprising the CH2 and CH3 domain and, optionally, at least a part of the hinge region. The immunoglobulin domain may be an IgG, IgM, IgD or IgE immunoglobulin domain or a modified immunoglobulin domain derived therefrom. Preferably, the second domain comprises at least a portion of a constant IgG immunoglobulin domain. The IgG immunoglobulin domain may be selected from IgG1, IgG2, IgG3 of IgG4 domains or from modified domains such as are described in US 5,925,734. The immunoglobulin domain may exhibit effector functions, particularly effector functions selected from ADCC and/or CDC. In some embodiments, however, modified immunoglobulin domains having modified, e.g. at least partially deleted, effector functions may be used.

Designing the fusion protein of the present invention comprises a selection of the terminal amino acid(s) of the first domain and of the second domain in order to create an at least one amino acid overlap between both domains. In order to achieve this goal it is usually necessary to delete one or several amino acids from a first and/or second domain and/or to add one or several amino acids from the naturally occurring adjacent domain to the first and/or second domain. For example, it may be necessary to provide a first domain having a deletion of preferably up to 10 and, more preferably, up to 6 amino

acids, e.g. 1, 2, 3, 4, 5 or 6 amino acids from naturally occurring domain boundaries. On the other hand, it may be required to add preferably up to 10 and, more preferably, up to 6 amino acids, e.g. 1, 2, 3, 4, 5 or 6 amino acids from a naturally occurring adjacent domain to the first and/or second domain. When deleting and/or adding amino acids, however, one has to take care that the biological activity of the first domain and/or the second domain is not detrimentally affected.

The fusion protein of the invention may comprise an N-terminal signal sequence which allows secretion from a host cell after recombinant expression. The signal sequence may be a signal sequence which is homologous to the first domain of the fusion protein. Alternatively, the signal sequence may also be a heterologous signal sequence, e.g. the Igκ or the Igλ signal peptide sequence. In a different embodiment, the fusion protein is free from an N-terminal sequence, thus representing the mature form of the fusion protein.

The overlap between the first and the second domain or between two first domains has a length of preferably 1, 2 or 3 amino acids. More preferably the overlap has a length of one amino acid. Examples of overlapping amino acids are S, E, K, H, T, P and D.

The present invention is explained in detail below with regard to several specific preferred embodiments. It should be noted, however, that further fusion proteins of the invention may be manufactured by analogous means.

In a first preferred embodiment the first domain is the extracellular domain of human CD95. The extracellular domain of the fusion protein preferably comprises the amino acid sequence up to amino acid 170, 171, 172 or 173 of human CD95. Preferably, the extracellular domain of CD95 is fused with a human IgG Fc fragment, e.g. a human IgG1 Fc fragment. The amino acid sequence of the human CD95 molecule is shown in Figure 1. The amino acid sequence of the human IgG1 chain constant domain is shown in Figure

2. Especially preferred is the fusion protein comprising the amino acid sequence as shown in Figures 3A and 3B, wherein the overlapping amino acid sequence is S.

In a further especially preferred embodiment the first domain is the extracellular domain of a human TRAIL receptor, e.g. the human TRAIL receptor-1, the human TRAIL receptor-2, the human TRAIL receptor-3 and the human TRAIL receptor-4. The extracellular domain preferably comprises the amino acid sequence up to amino acid 232, 233, 234, 235, 236, 237, 238, 239 (TRAILR-1), 204, 205, 206, 207, 208, 209, 210 (TRAILR-2 long), 185, 186, 187, 188, 189, 190, 191 (TRAILR-2 long – without repeat), 179, 180, 181, 182, 183, 184 (TRAILR-2 short), 228, 229, 230, 231, 232, 233, 234, 235, 236, (TRAILR-3), 151, 152, 153, 154, 155, 156, 157, 158, 159, 160, 161 (TRAILR-3 without repeat) and 201, 202, 203, 204, 205, 206, 207, 208, 209, 210, 211 (TRAILR-4). Especially preferred is the human TRAIL receptor-2. The extracellular human TRAIL receptor domain may be fused with a human IgG-1 Fc fragment. The amino acid sequences of human TRAIL receptors are shown in Figure 4 (TRAILR-1), Figure 6 (TRAILR-2 long), Figure 9 (TRAILR-2 short), Figure 11 (TRAIL-3) and Figure 14 (TRAILR-4). Specific examples of preferred fusion proteins comprise amino acid sequences as shown in Figure 5, 7, 8, 10, 12, 13 and 15.

In still a further preferred embodiment the fusion protein comprises a first domain which is the extracellular domain of a human TNF receptor, e.g. a human TNF receptor-1 or a human TNF receptor-2. The extracellular domain preferably comprises the amino acid sequence up to amino acid 203, 204, 205, 206, 207, 208, 209, 210, 211 (TNF-R1) or 248, 249, 250, 251, 252, 253, 254, 255, 256, 257 (TNF-R2). The extracellular domain of the human TNF receptor may be fused to a human IgG-1 Fc fragment. The amino acid sequence of human TNF receptors are shown in Figures 16 (TNF-R1) and 18 (TNF-R2). Specific examples of preferred fusion protein comprise amino acid sequences as shown in Figures 17 and 19.

A further aspect of the present invention relates to a nucleic acid molecule encoding a fusion protein as described above. The nucleic acid molecule may be a DNA molecule, e.g. a double-stranded or single-stranded DNA molecule, or an RNA molecule. The nucleic acid molecule may encode the fusion protein or a precursor thereof, e.g. a pro- or pre-proform of the fusion protein which may comprise a signal sequence or other heterologous amino acid portions for secretion or purification which are preferably located at the N- and/or C-terminus of the fusion protein. The heterologous amino acid portions may be linked to the first and/or second domain via a protease cleavage site, e.g. a Factor X_a, thrombin or IgA protease cleavage site.

The nucleic acid molecule may be operatively linked to an expression control sequence, e.g. an expression control sequence which allows expression of the nucleic acid molecule in a desired host cell. The nucleic acid molecule may be located on a vector, e.g. a plasmid, a bacteriophage, a viral vector, a chromosal integration vector, etc. Examples of suitable expression control sequences and vectors are described for example by Sambrook et al. (1989) *Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor Press, and Ausubel et al. (1989), *Current Protocols in Molecular Biology*, John Wiley & Sons.

Various expression vector/host cell systems may be used to express the nucleic acid sequences encoding the fusion proteins of the present invention. Suitable host cells include, but are not limited to, prokaryotic cells such as bacteria, e.g. *E.coli*, eukaryotic host cells such as yeast cells, insect cells, plant cells or animal cells, preferably mammalian cells and, more preferably, human cells.

Further, the invention relates to a non-human organism transformed or transfected with a nucleic acid molecule as described above. Such transgenic organisms may be generated by known methods of genetic transfer including homologous recombination.

A further aspect of the present invention relates to a pharmaceutical composition comprising as an active agent at least one fusion protein or a nucleic acid molecule coding thereof as described above. In an especially preferred embodiment, the first domain is a soluble death receptor, e.g. the extracellular domain of a death receptor as described above for use in the prophylaxis and/or treatment of disorders associated with apoptosis. Most preferably, the first domain is the extracellular CD95 domain.

In this embodiment of the invention the composition may be used in the prophylaxis and/or treatment of disorders selected from autoimmune disorders, AIDS, heart disorders, e.g. myocardial infarction, graft-versus-host-disorders, transplant rejection, brain damage, e.g. stroke, spinal cord injuries, e.g. paraplegia, sepsis, hepatitis, disorders associated with inflammation, ischemic reperfusion injury and renal disorders. These disorders and further disorders which may be treated by administration of death receptor fusion proteins, particularly CD95 fusion proteins, are described in WO 95/27735, WO 99/50413, WO 01/41803, EP-A-0 965 637 and EP-A-0 992 243 which are herein incorporated by reference.

The fusion protein is administered to a subject in need thereof, particularly a human patient, in a sufficient dose for the treatment of the specific conditions by suitable means. For example, the fusion protein may be formulated as a pharmaceutical composition together with pharmaceutically acceptable carriers, diluents and/or adjuvants. Therapeutic efficacy and toxicity may be determined according to standard protocols.The pharmaceutical composition may be administered systemically, e.g. intraperitoneally, intramuscularly or intravenously or locally, e.g. intranasally, subcutaneously or intrathecally. Preferred is intravenous administration.

Especially preferred is a death ligand inhibitor, e.g. a soluble extracellular CD95 or TRAIL receptor domain fused to an Fc fragment.

The dose of the fusion protein administered will of course be dependent on

the subject to be treated, on the subject's weight, the type and severity of the injury, the manner of administration and the judgement of the prescribing physician. For the administration of CD95 or TRAIL-R fusion proteins, a daily dose of 0,001 to 100 mg/kg is suitable.

Moreover, the invention relates to a method for manufacturing a fusion protein comprising

(i) at least one first domain comprising a biologically active protein fused to

(ii) a second domain comprising at least a portion of a constant immunoglobulin domain with reduced immunogenic potential, wherein the first domain is fused to the second domain with at least one amino acid overlap.

Still a further aspect of the present relates to a fusion protein comprising:

(i) at least one first domain comprising a biologically active polypeptide fused to

(ii) a heterologous second domain which is capable of oligomerising the fusion protein wherein there is at least one amino acid overlap between the first and the second domain in the fusion region.

Fusion proteins comprising heterologous second domains which are capable or oligomerising the fusion proteins in the absence of third proteins are described in WO 01/49866 and in WO 02/090553, for example, which are incorporated herein by reference. The presence of at least one amino acid overlap, e.g. one, two or three amino acids overlap, between the first and the second domain in the fusion proteins leads – as explained above – to fusion proteins with reduced immunogenic potential.

The first domain in this oligomerising fusion protein is defined as above. Preferably, the first domain is an extracellular domain of a membrane-anchored receptor, or a ligand-binding fragment thereof. Especially preferred

is that the receptor is selected from CD95, a TRAIL receptor, particularly the TRAIL receptor-2 and a TNF receptor, particularly the TNF receptor-2. Alternatively, the first domain may be a receptor-binding ligand domain, wherein the ligand is preferably selected from CD95 ligand, TRAIL and TNF. Specific examples of preferred first domains are as described above.

The second domain of the fusion protein comprises an oligomerising portion of a protein. Preferably, the second domain is capable of di- tri- tetra- or pentamerising the fusion protein. In this context, particular reference is made to the disclosure of WO 01/49866 and WO 02/090553, which are herein incorporated by reference. Preferred examples of second domains are C1q, MBP (Mannose Binding Protein), SP-A (Lung Surfactant Protein-A), SP-D (Lung Surfactant Protein-D), BC (Bovine Serum Conglutinine), CL43 (Bovine Collectine-43), ACRP-30 (a protein from the C1q family) and COMP (Cartilage Oligomeric Matrix Protein) or the collagen domain of EDA or a functionally active derivative thereof. Especially preferred are portions of ACRP--30, particularly of the human ACRP-30 protein, e.g. amino acids 18 to 108, or 18 to 110 or of COMP.

As described above, the first domain(s) of the fusion protein may be located at the N- or C-terminus and the second domain at the C- or N-terminus. Further, both the first and the second domains are preferably from the same species, more preferably of human origin. Furthermore, the features relating to preferred embodiments of the fusion proteins based on immunoglobulins also apply to the oligomerising fusion proteins.

The reduced immunogenic potential of the fusion protein results from the lack of non-naturally occurring transitions between the first and the second domain in the fusion proteins, which in turn leads to a decreased potential for the formation of neo-epitopes resulting from the fusion between two heterologous polypeptides.

The present invention is illustrated further by the following Figures and Examples.

Figure Legend

Figure 1: the amino acid sequence of the human CD95 (APO-1; Fas) protein;

Figure 2: the amino acid sequence of the human IgG-1 chain C-region;

Figures 3A and 3B: a preferred example of a CD95-Fc IgG1 fusion protein with an overlapping amino acid;

Figure 4: the amino acid sequence of the human TRAIL receptor-1;

Figure 5: preferred examples of TRAILR-1 Fc IgG1 fusion proteins with overlapping amino acids;

Figure 6: the amino acid sequence of human TRAIL receptor-2 (long form);

Figure 7: preferred examples of TRAILR-2 (long) Fc IgG1 fusion proteins with overlapping amino acids, including a repeat sequence;

Figure 8: preferred examples of TRAILR-2 (long form) Fc fusion proteins with overlapping amino acids (without repeat sequence);

Figure 9: the amino acid sequence of human TRAILR-2 (short form);

Figure 10: preferred examples of TRAILR-2 (short) Fc IgG1 fusion proteins with overlapping amino acids;

Figure 11: the amino acid sequence of human TRAIL receptor R-3;

Figure 12: preferred examples of TRAILR-3 Fc IgG1 fusion proteins with overlapping amino acids (repeats included);

Figure 13: preferred examples of TRAILR-3 Fc IgG1 fusion proteins with overlapping amino acids (repeats not included);

Figure 14: the amino acid sequence of human TRAIL receptor-4;

Figure 15: preferred examples of TRAILR-4 Fc IgG1 fusion proteins with overlapping amino acids;

Figure 16: the amino acid sequence of human tumor necrosis factor receptor-1;

Figure 17: preferred examples of TNFR-1 Fc IgG1 fusion proteins with overlapping amino acids;

Figure 18: the amino acid sequence of human tumor necrosis factor

receptor-2;

Figure 19: preferred examples of TNF-R2 Fc IgG1 fusion proteins with overlapping amino acids.

Example 1

Fusion protein consisting of the human CD95 extracellular domain and the human IgG1 Fc domain with overlapping amino acids.

Human CD95 extracellular domain

Bases 221-736 of Human CD95 (Genbank Acc. No. X63717). Utilized Sequence from Oehm, A., "Purification and Molecular Cloning of the APO-1 Cell Surface Antigen, a Member of the Tumour Necrosis Factor/Nerve Growth Factor Receptor Superfamily," Journal of Biological Chemistry Vol.267, No.15, pp.10709-10715, 1992. cDNA was created from total RNA isolated from Peripheral Blood Lymphocytes (PBL) from donor blood by RT-PCR using Oligo dT primer. PCRs were used to amplify the cDNA of the extracellular domain of CD95 by including a restriction Hind III Site and a Kozak Sequence at the 5' of the Extracellular domain and at the 3' a Bgl II site (termination of the extracellular domain).

PCR primers for the amplification of CD95 cDNA with Taq polymerase:
Sense huCD95-Hind III: TATA AAGCTT GCC ACC ATG CTG GGC ATC TG (SEQ ID NO:21)
Antisense huCD95-Bgl II: TATA AGATCT GGA TCC TTC CTC TTT GC (SEQ ID NO:2)

Human IgG1 Fc domain

Sequence: 2050-2745 bp. Sequence used from, Ellison, J., "The nucleotide sequence of human immunoglobulin C gene", Nucleic Acid Research, Volume 10 Number 13, 1982. cDNA was created from total RNA isolated from Peripheral Blood Lymphocytes (PBL) from donor blood by RT-PCR using Oligo dT primer. A PCR was used to amplify the cDNA of human IgG1

Fc (partial hinge-CH3) by including a restriction Bgl II site at the 5' of the primer and at the 3' primer after the stop codon, an Xho I site.

PCR primers for the amplification of IgG1 Fc cDNA with Taq polymerase:
Sense huIgG1Fc-BglII: TATA AGATCT TGT GAC AAA ACT CAC ACA TG (SEQ ID NO: 3)
Antisense huIgG1Fc-XhoI: TATA CTCGAG TCA TTT ACC CGG AGA CAG GG (SEQ ID NO: 4)

Cloning Procedure:

Following amplification the IgG1 Fc PCR product was digested with Bgl II and Xho I. The CD95 PCR product was digested with Hind III and Bgl II and pcDNA3.1 (with CMV promoter) with Hind III and Xho I. The products were purified via gel extraction (Qiagen Kit).

The huIgG1Fc and CD95 fragments were ligated with T4 ligase into pcDNA3.1. After transfection of One Shot Top 10 chemically competent cells (E.coli) from Invitrogen Ordering # C4040-10 and amplification, a plasmid preparation was performed with Qiagen Plasmid Prep Kit.

A three point ligation was performed by digesting pcDNA3.1 with HindIII and XhoI, CD95EC with HindIII and BglII, and huIgG1 Fc with BglII and XhoI. The presence of the CD95-huIgG1 Fc insert in pcDNA3.1 was verified by sequencing and restriction enzyme analysis. The vector containing insert was digested with HindIII and XbaI and the insert was ligated into pcDNA3.1 containing the EF-1 promoter.

The Kozak sequence of the original CD95-Fc construct was changed from GCCACCATGC to GCCGCCACCATGG by amplification of the whole CD95-Fc product with the primers SEQ ID 5 and SEQ ID 6.

Primers for Changing the Kozak Sequence from GCCACCATGC to GCCGCCACCATGG:

ShuCD95EC_altKozak TATA AAGCTT GCC GCC ACC ATG GTG GGC ATC (SEQ ID NO. 5)

AS698 huIgG1Fc-Xho1 TATA CTCGAG TCA TTT ACC CGG AGA CAG GG (SEQ ID NO:6)

Cloning Procedure:

The PCR product was cloned in pcDNA3.1/V5 His Topo vector from Invitrogen (Ordering # K4800-01), digested with Hind III and Xba I as well as pcDNA3.1 containing the pEF promoter and ligated with T4 Ligase.

Expression and Isolation

The construct encoding the final product was transfected into cell lines suitable for protein expression. Transfection can be performed by any standard method know to those skilled in the art. Examples include electroporation, liposomal mediated transfer, calcium phosphate transfection. Cell lines suitable for the expression include 293T cells, COS-1, COS-7 and CHO cells. Other cell lines may be used.

In this example, 293T cells were transiently transfected by the calcium phosphate method. Alternatively, CHO cells were transfected utilizing FuGene6 and stable clones were selected.

The desired protein can be purified from the cell culture medium by chromatographic methods. Methods include but are not limited to affinity chromatography on protein-G or protein-A columns, ion-exchange chromatography, hydrophobic interaction chromatography, size exclusion chromatograpy or a combination of these methods.

In the example the supernatant was purified on IgG columns (Amersham Pharmacia) according to the manufacturers instructions, leading to a higly purified product in a single step.

Example 2.

Fusion protein consisting of the TRAIL receptor-2 and the human IgG1 Fc domain with overlapping amino acids

Human IgG1 Fc domain:

Sequence used from, Ellison, J., "The nucleotide sequence of human immunoglobulin C gene", Nucleic Acid Research, Volume 10 Number 13, 1982. cDNA was created from total RNA isolated from Peripheral Blood Lymphocytes (PBL) from donor blood by RT-PCR using Oligo dT primer. A PCR was used to amplify the cDNA of human IgG1 Fc (partial hinge-CH3) with an overlapping sequence to TRAILR2 at the 5' end and at the 3' end after the stop codon an EcoRI site.

I. Primer: **Sense_huIgG1** (SEQ ID NO: 7)
cca ggg act cct gcc TCT TGT GAC AAA ACT CAC ACA TG (Capital letters => part of huIgG1)

II. Primer: **Antisense_ERIhuIgG1** (SEQ ID NO: 8)
TATA gaa ttc tca ttt acc cgg aga cag gg

TRAILR2:

Utilized Sequence from Walczak H., "TRAIL-R2: a novel apoptosis-mediating receptor for TRAIL" The EMBO Journal Vol.16, No.17, pp.5386-5397, 1997. (Accession number DDBJ/EMBL/GenBank: AF016849) cDNA was created from total RNA isolated from Peripheral Blood Lymphocytes (PBL) from donor blood by RT-PCR using an Oligo dT primer. A PCR was used to amplify the cDNA of TRAILR2 domain by including a restriction site Hind III and a Kozak Sequence at the 5' end and at the 3' end an overlapping sequence to human IgG1.

III. Primer: **Sense_HIII_TRAILR2** (SEQ ID NO: 9)
TATA aag ctt gcc gcc acc atg gaa caa cgg gga cag aac

IV. Primer: **Antisense_TRAILR2** (SEQ ID NO: 10)
gtg agt ttt gtc aca aga GGC AGG AGT CCC TGG (Capital letters => part huTRAIL-R2, in reverse)

Cloning Procedure:
Following the amplification a gel extraction was performed to isolate the modified inserts. Then a third PCR utilizing both fragments was performed. Due to the overlap of both fragments and the primers at the end, this PCR joins in one product. Afterwards the product was digested with Hind III and EcoR I and ligated in a suitable expression vector, e.g. pcDNA3.1 (Invitrogen).

III. Primer: **Sense_HIII_TRAILR2** (SEQ ID NO: 11)
TATA aag ctt gcc gcc acc atg gaa caa cgg gga cag aac

II. Primer: **Antisense_ERIhuIgG1** (SEQ ID NO: 12)
TATA gaa ttc tca ttt acc cgg aga cag gg

Expression
The construct was cloned and expressed in suitable host cells as described in Example 1.

Example 3.

Use of a CD95-Fc construct for the regeneration and functional recovery after spinal cord injury.

The CD95-Fc construct with overlapping amino acids as described in Example 1 was used for the treatment of spinal cord-injury in a mouse model as described by Demjen et al., *Nat Med.* (March 7, 2004). It was found that administration of the construct promotes regeneration and functional recovery after spinal cord injury.

Example 4.

Use of CD95-Fc construct for the attenuation of brain damage in stroke.

The CD95-Fc construct with overlapping amino acids was investigated for its influence on primary ischemic death and secondary inflammatory injury in a mouse model as described by Martin-Villalba et al. (*Cell Death Differ.* 8 (2001), 679-686). It was found that administration of the CD95-Fc construct resulted in a significant decrease in both infarct volumes and mortality.